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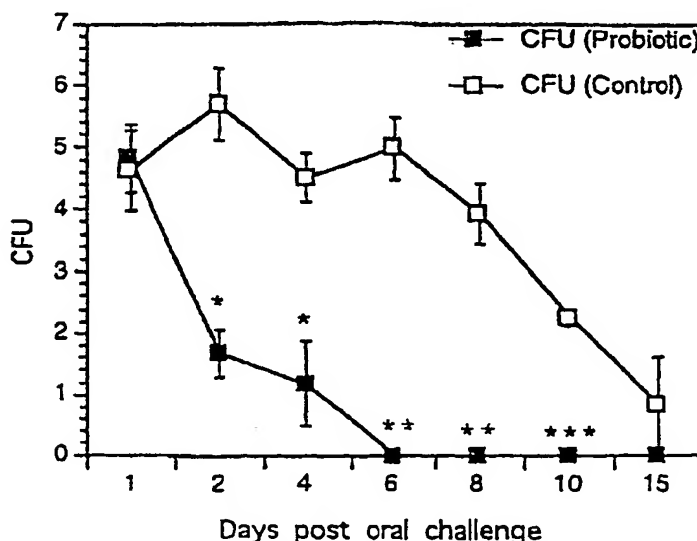
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(57) Abstract: Compositions and methods for therapeutic or prophylactic treatment of disorders associated with mucosal surfaces and in particular to treatment of infectious disorders at mucosal sites by enhancing non-specific mucosal immunity, especially with probiotics such as lactobacillus or mycobacterium vaccae.

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**IMMUNOTHERAPY OR TREATING BACTERIAL OR VIRAL INFECTION AT MUCOSAL  
SURFACES WITH PROBIOTICS, AND COMPOSITIONS THEREFOR**

**TECHNICAL FIELD**

The present invention relates to compositions and methods suitable for therapeutic or prophylactic treatment of diseases associated with mucosal  
5 surfaces and in particular to treatment of infectious disorders at mucosal site by way of enhancing non-specific mucosal immunity.

**BACKGROUND ART**

The last 30 years has witnessed an explosive increase in understanding of the mechanisms of mucosal protection, beginning with the recognition that  
10 mucosal immunity was partitioned from systemic immunity (with IgA as a marker), that it was driven from the gut-associated lymphoid tissue (specifically Peyer's patches), that it involved both T and B lymphocytes and that a specific recirculation of gut-derived lymphocytes between the mucosal surfaces ensured participation of all mucosal surfaces in responses generated by delivery of  
15 antigen to the Peyer's patch. Early studies focused on IgA, but gradually the key role played by T lymphocytes and the cytokines they secrete, have dominated thinking. The concept of "cytokine profiles" became important as it was shown that T cells could be characterised by the particular pattern of cytokines secreted, leading to the concept of Th1 and Th2 CD4+ve T cells. Th1  
20 cells secreted IFN- $\gamma$  while Th2 cells were characterised by IL-4 secretion. This "pattern" determined outcome and now many infection outcomes are known to be influenced by the pattern of cytokines secreted.

The above focuses on specific immunity initiated by particular antigens. The non-specific immune response "sits" on, and operates through, an array of  
25 cells and molecules which are powerful effector mechanisms operating without the specificity gained through antigen receptors.

The value in health promotion of a range of gut microbes (probiotics) taken in a variety of food or formulation forms has been recognised for some time. Claims to the value of these probiotics are mainly non-specific and without  
30 scientific support, and are largely based on clinical impression. The probiotics are thought to promote health via reconstitution of what is presumed to be beneficial normal flora. Use of probiotics for treatment of certain specific

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intestinal conditions has been reported. However, the frequently exaggerated claims serve to reduce credibility rather than consolidate therapeutic benefits.

Changing patterns of proneness to mucosal disease (eg. allergy, infection) are increasingly being linked to microbe/gut mucosal "experience", in areas which include changing incidence of allergy and asthma linked to environment "sterility" and altered gut bacterial flora, and reduced mucosal infection and allergy in infants given avirulent *E. coli*.

There is therefore a need for improved preparations and methods for prophylaxis or therapy of mucosal disorders such as infections, allergy and the like. There is also a need for preparations and methods which can be used for prevention of reactivation of, or reinfection with, viruses and for the treatment of conditions and syndromes associated with viral infection or reinfection.

It is an object of the present invention to overcome or ameliorate at least some of the disadvantages of the prior art, or to provide a useful alternative.

15

#### **SUMMARY OF THE INVENTION**

The present invention is in part based on the observation that certain microorganisms, in particular lactobacilli, can prime the mucosal surfaces by inducing a particular cytokine "pattern", thus creating conditions unfavourable to microbial colonisation, and/or microbial pathogenesis, and in part on the novel demonstration of non-antigen activated cells migrating within the common mucosal system.

The microorganisms useful in the practice of the present invention may or may not have traditional probiotic effects, but they will be able to alter the cytokine pattern or balance, or induce a Th1-type cellular response. For convenience the useful microorganisms or their components may also be referred to herein as probiotics, whether or not they in fact have a probiotic effect. It is intended that this term ("probiotic") includes in its scope other adjuvant agents capable of inducing a Th1-type cellular response.

According to a first aspect there is provided a method of prophylactic or therapeutic treatment of chronic or acute infection, or of undesirable microbial colonisation, of a mucosal surface, other than intestinal mucosal surface, comprising the administration of an effective amount of a probiotic, or a probiotic-containing composition, to a subject in need thereof.

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In this embodiment of the invention the mucosal surface is preferably selected from the group consisting of oral, nasopharyngeal, respiratory, gastric, reproductive and glandular. The infection or colonisation can be acute or chronic and may be bacterial, fungal or viral. It will be understood that chronic  
5 viral infection will include certain syndromes which may have origins in viral infection, such as for example chronic fatigue syndrome and the like.

According to a second aspect there is provided a method of prophylactic or therapeutic treatment of a chronic or acute disorder of mucosal surface of the respiratory tract, comprising the administration of an effective amount of a  
10 probiotic, or a probiotic-containing composition, to a subject in need thereof.

Preferably the respiratory tract mucosal surface is the upper respiratory tract mucosal surface and even more preferably it is oral or lung mucosa.

Preferably the probiotic, or a probiotic-containing composition, is administered to the gastric or to the intestinal mucosal surface.

15 According to a third aspect there is provided a method of prophylactic or therapeutic treatment of a chronic or acute disorder of a mucosal surface caused by disturbance in cytokine balance or lack of an appropriate T cell immune response, comprising the administration of an effective amount of a probiotic, or a probiotic-containing composition, to a subject in need thereof.

20 Preferably the mucosal surface is selected from the group consisting of oral, nasopharyngeal, respiratory, gastric, intestinal, reproductive and glandular.

A useful marker for assessment of cytokine balance is one or more of interferon- $\gamma$  (IFN- $\gamma$ ), interleukin-4 (IL-4) and interleukin-12 (IL-12). However, other cytokines known as markers for either Th1 or Th2 cellular responses are  
25 also useful for this purpose.

It is preferred that the probiotic is a bacterium, for example one which can be selected from, but not limited to, lactic acid bacteria, *Mycobacterium* species or *Bifidobacterium* species. Even more preferred is the use of *Lactobacillus acidophilus* (*L. acidophilus*), *Lactobacillus fermentum* (*L. fermentum*) or  
30 *Mycobacterium vaccae* (*M. vaccae*), or parts thereof which are capable of inducing the Th1 cellular response. Specially preferred is *L. acidophilus*. *L. acidophilus*, *L. fermentum* or *M. vaccae* may be used live or as an inactivated preparation, as long as they are capable of inducing the Th1 response. For

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preference *L. acidophilus* and *L. fermentum* is used as a live preparation. It is considered that other bacteria would also be suitable as probiotics as herein defined (whether they have probiotic effect or not), for example the well known adjuvating bacteria such as for example *L. casei*, *L. plantarum*, *L. rhamnosus*,  
5 *Bifidobacterium breve* and the like.

The required dosage amount will vary according to the nature of the mucosal surface disorder, whether used prophylactically or therapeutically and the type of organism or neoplasm involved. The treatment parameters as well as the required dosage can be easily assessed by those skilled in the art. The  
10 preferred dosage of the probiotic, when the probiotic is a whole live probiotic bacterium, is from about  $1 \times 10^8$  to about  $1 \times 10^{12}$  organisms.

The probiotic may be administered in conjunction with one or more antibiotics or one or more other pharmaceutically active agents. The probiotic may be administered prior to, simultaneously with or subsequent to antibiotic  
15 therapy or therapy with other active agents.

Preferably, the mucosal surface disorder is a bacterial infection such as for example infection by *Pseudomonas* species, *Streptococcus* species, *Staphylococcus* species, *Candida* species, *Helicobacter* species or *Haemophilus* species. Even more preferred are non-typable *Haemophilus*  
20 *influenzae*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Staphylococcus albus*, *Staphylococcus aureus*, *Candida albicans* and/or *Helicobacter pylori*. The mucosal disorder may also be inappropriate colonisation by bacteria or other microorganisms. For example the mucosal disorder may involve chronic or acute colonisation or infection by viruses such  
25 as Epstein-Barr virus (EBV), cytomegalovirus (CMV), Herpes viruses and the like. It would be clear to those skilled in the art that the treatment of infections and/or colonisation by other viruses can also be achieved with the methods and compositions of the present invention.

Preferably, the subject in need of treatment is selected from the group  
30 consisting of individuals having high risk of infection. However, it will be recognised that the present treatments are suitably employed in prophylaxis of mucosal disorders in any subject. For example, the treatment methods of the present invention may be suitably administered to subjects exposed to a variety

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of stressors which have an impact on the subjects immune status and thus predisposes them to infection. For example fatigue and/or physical stressors such as commonly encountered by athletes, predisposes these subjects to respiratory tract infections. This seems to be caused by impairment in the  
5 immune status of mucosal surfaces, in particular the secretion of IgA.

Administration of the probiotic preparations, such as those described herein, prior to, during and/or after exercise or training may restore their immune status, particularly that of the mucosal surfaces of the respiratory tract, thus combating or preventing infection.

10 Preferably, the probiotic or probiotic-containing composition is in tablet or capsule form. However, it will be clear to those skilled in the art that the probiotic composition may be in a liquid or other forms of solid preparations and may also be present in a food source such as a yoghurt or other dairy product, or similar non-dairy products based for example on soy..

15 The treatment may involve administration of a probiotic, or of a probiotic-containing composition, to a site which is distal to the mucosal surface having the disorder. For example the probiotic, or the probiotic-containing composition, can be administered to the intestinal mucosal surface in the treatment of nasopharyngeal, gastric or upper respiratory tract mucosal infection.

20 According to a fourth aspect there is provided a method of altering cytokine balance at a mucosal surface in a subject comprising the administration of an effective amount of a probiotic, or of a probiotic-containing composition, to a subject in need thereof.

According to a fifth aspect there is provided a method of inducing a Th1  
25 cellular immune response at a mucosal surface in a subject comprising the administration of an effective amount of a probiotic, or of a probiotic-containing composition, to a subject in need thereof.

According to a sixth aspect there is provided a method of enhancing the secretion of interferon- $\gamma$  at a mucosal surface in a subject comprising the  
30 administration of an effective amount of a probiotic, or of a probiotic-containing composition, to a subject in need thereof.

According to a seventh aspect there is provided a method of reducing the secretion of interleukin-4 at a mucosal surface in a subject comprising the

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administration of an effective amount of a probiotic, or of a probiotic-containing composition, to a subject in need thereof.

According to an eighth aspect there is provided a method of restoring normal cytokine balance at a mucosal surface in a subject comprising the  
5 administration of an effective amount of a probiotic, or of a probiotic-containing composition, to a subject in need thereof.

According to a ninth aspect there is provided a method of priming mucosal surface for immunotherapy comprising the administration of an effective amount of a probiotic, or of a probiotic-containing composition, to a  
10 subject in need thereof.

Preferably the immunotherapy consists in administration of a therapeutic or prophylactic vaccine. Also preferred is a procedure whereby the probiotic, or the probiotic-containing composition, is administered before or simultaneously with the vaccine. The administration of the probiotic, or of the probiotic-  
15 containing composition, may be continued for a period after the vaccine has been administered.

According to a tenth aspect there is provided a pharmaceutical composition suitable for prophylactic or therapeutic treatment of chronic or acute disorder of a mucosal surface comprising an effective amount of a probiotic, or  
20 of a probiotic-containing composition.

It is preferred that the probiotic is a bacterium, for example one which can be selected from, but not limited to, lactic acid bacteria, *Mycobacterium* species or *Bifidobacterium* species. Even more preferred is the use of *Lactobacillus acidophilus* (*L. acidophilus*), *Lactobacillus fermentum* (*L. fermentum*) or  
25 *Mycobacterium vaccae* (*M. vaccae*), or parts thereof which are capable of inducing the Th1 cellular response. Specially preferred is *L. acidophilus*. *L. acidophilus*, *L. fermentum* or *M. vaccae* may be used live or as an inactivated preparation, as long as they are capable of inducing the Th1 response. For preference *L. acidophilus* and *L. fermentum* is used as a live preparation. It is  
30 considered that other bacteria would also be suitable as probiotics as herein defined (whether they have probiotic effect or not), for example the well known adjuvating bacteria such as for example *L. casei*, *L. Plantarum*, *L. rhamnosus*, *Bifidobacterium breve* and the like.



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Preferably the pharmaceutical composition includes viable organisms, however it will be understood that killed organisms may also be used. Further, single organism preparations or preparations containing multiple organisms are envisaged. The organism(s) can be either intact or disrupted.

5       The preferred content of the probiotic, when the probiotic is a whole live probiotic bacterium, is one that will deliver a dosage from about  $1 \times 10^8$  to about  $1 \times 10^{12}$  organisms.

      The preferred formulation of the composition is in a solid dosage form, such as for example tablet or capsule. It will be understood however that it may  
10   be formulated in the form of a food product, for example soy-based or dairy-based product.

      According to an eleventh aspect there is provided a method of prophylactic or therapeutic treatment of a symptom and/or syndrome associated with chronic or acute infection, or with undesirable microbial colonisation or reactivation, of a  
15   mucosal surface, comprising the administration of an effective amount of an agent capable of non-specific activation of the common mucosal system to a subject in need thereof.

      Preferably the symptom and/or syndrome to be treated is chronic fatigue syndrome.

20       The preferred agent is a probiotic, or a probiotic-containing composition. Also preferred is that the probiotic is, or the probiotic-containing composition comprises, viable intact organisms.

      Preferably the subject to be treated is selected from the group consisting of an EBV positive athlete, a subject with sudden onset CFS, a subject with  
25   protracted fatigue following exercise, a subject with low level of salivary IgA or IgA1 and a subject with documented infectious mononucleosis.

#### BRIEF DESCRIPTION OF FIGURES

Figure 1: IL-4 and IFN- $\gamma$  production following feeding with *Lactobacillus acidophilus*

30   Figure 2: Secretion of NO into saliva of animals fed *Lactobacillus acidophilus* following challenge with *Candida albicans*

Figure 3: Effect of probiotic on IL-12 production following challenge with *Candida albicans*

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Figure 4: Effect of probiotic on IL-4 production following challenge with *Candida albicans*

Figure 5: Resistant to infection in animals fed *L. acidophilus*.

Figure 6: Effect of probiotic on salivary IFN- $\gamma$  following challenge with  
5 *Candida albicans*

Figure 7: Effect of probiotic on cervical lymph node IFN- $\gamma$  following challenge with *Candida albicans*

Figure 8: Salivary IFN- $\gamma$  following challenge with *C. albicans*.

Figure 9: Effect of treatment with L-MLNA on clearance of *C. albicans* in  
10 mice

### DESCRIPTION OF THE PREFERRED EMBODIMENTS

The current invention is about the manipulation of cells and molecules that participate in both these systems, to "prime" mucosal surfaces to produce Th1 cytokines in response to "danger". The concept that is developed is that certain  
15 bacteria, specially lactobacilli, populate the gut of subjects exposed to high density pathogens. Our studies have shown that these bacteria influence the cytokine pattern towards a Th1 response. The activated and IFN- $\gamma$  producing T cells populate the mucosal surfaces and their regional lymph nodes, setting up a containing armament awaiting "danger".

20 The methods and compositions of the present invention can also be used effectively in the treatment of acute and chronic viral infections. In particular the treatment of chronic Epstein-Barr virus (EBV), cytomegalovirus (CMV) and other herpes-type virus infection, which are ubiquitous in the population and are associated with numerous symptoms and diseases, are envisaged as particularly  
25 useful applications. Also envisaged as a useful application of the compositions and methods of the present invention is the treatment of disorders which may have origins in or are triggered by viral infections which are prevalent in the population and which can be debilitating but have no known or no well defined treatment protocol, such as for example chronic fatigue syndrome ("CFS").

30 Substantial number of patients currently diagnosed as "CFS" reflect repeatedly activated EBV (or similar herpes group virus infection), due to

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impaired mucosal immunity. This can be monitored through measurement of salivary IgA1 or INF- $\gamma$ , or PCR detected free virus. Characteristic (but not limiting) clinical features of those likely to respond to probiotic therapy includes onset of "viral illness", leaving a state of chronic relapsing fatigue. Acute  
5 deterioration occurs with "infection", usually noted as a sore throat, or excessive exercise.

Viral infection need not cause immediately recognisable symptoms and may be dormant in otherwise fit and healthy subjects for a significant periods of time, but may reactivate once a trigger is provided. For example, in athletes  
10 undergoing intensive training or exercise impaired performance may be caused by reduced mucosal containment of EBV, which leads to reactivation and excretion of virus, which in turn leads to impaired performance and/or fatigue. Thus, athletes who are EBV positive and have impaired performances, specially if low salivary IgA or IgA1 is detected (for example less than 50 mg/100ml),  
15 could benefit from treatment with preparations of the present invention.

Of course the preparations and methods of the present invention can also be applied to other subjects, for example patients diagnosed as "CFS", where a virus (usually EBV but can be CMV, Ross River virus and the like) is reactivated and the fatigue is particularly initiated by exercise. However, any  
20 patient with documented infectious mononucleosis not getting better within several weeks and left with fatigue, any patient with protracted fatigue following clinical ( $\pm$  serological) evidence of viral illness or subjects with sudden onset CFS, with recurrent sore throats, with significant exacerbation of fatigue following exercise, or with low level of salivary IgA or IgA1 (eg. less than 50  
25 mg/100ml), could benefit from treatment with the preparations of the present invention.

It is envisaged that the administration of the probiotic or compositions containing the probiotic, will also assist in the treatment of symptoms and/or disorders described above in relation to viral infections.

30 The probiotics have the effect on mucosal surfaces distal to the site of administration of the probiotic. Thus, in broad terms the present invention is concerned with a probiotic product, in particular a product which is or includes lactobacilli, but may include other bacteria or combinations of bacteria, or

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indeed other adjuvants capable of inducing a Th1-type cellular response, which can be administered as a bolus or preferably regularly fed to maintain optimal mucosal protection of all mucosal surfaces through priming of mucosal T cells or maintaining the primed state of the cells. Such T cells contain INF $\gamma$  and can be  
5 triggered to release INF- $\gamma$  by non specific mechanisms. Therefore, other agents capable of activating non-specifically the common mucosal system may also be advantageously used in the preparations and methods of the present invention.

Further, the probiotics of the present invention may be used in  
10 conjunction with other treatments, to enhance or assist in their efficacy. For example, approximately 20% of patients treated with antibiotics for *H. pylori* infection fail to eradicate the organism. This resistance to antibiotic therapy may be due to a shift towards a Th0 response (ie, less IFN- $\gamma$  and more IL-4). Administration of a probiotic prior to, in conjunction with or subsequent to  
15 antibiotic therapy can be beneficial by switching back to a more dominant Th1 response and thus supplementing or assisting the antibiotic therapy to eradicate the organism in such patients.

Administration of probiotic can therefore be used for prevention or therapy of mucosal infections, colonisation of mucosal surfaces with abnormal  
20 or inappropriate organisms and reinfection with or reactivation of viruses.

The invention will now be described more particularly with reference to non-limiting examples.

## EXAMPLES

### **Example 1: Effect of probiotic bacteria on Th1/Th2 cytokine response**

25 To determine whether probiotic bacteria down-regulate Th2 and up-regulate Th1 cytokine response, C57/Bl6 mice were fed intragastrically using a feeding needle, various numbers of *Lactobacillus acidophilus* (obtained from University of New South Wales, School of Microbiology and Immunology Culture Collection, Sydney, Australia) on consecutive days for 2 weeks after which they  
30 were sensitised with 8  $\mu$ g of ovalbumin (OVA) and aluminium hydroxide in 0.2 mL phosphate-buffered saline administered by peritoneal injection. The mice

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were further fed ten times with *L. acidophilus* every two days for two weeks before they were sacrificed. Lymphocytes were isolated by teasing spleens through a sieve, washed with PBS, and resuspended at  $10 \times 10^6$ . One mL aliquots of the cell suspension were dispensed into wells of a 24-well flat-bottomed microtitre plate and stimulated with OVA ( $5 \mu\text{g/mL}$ ). After incubation for 4 days the supernatants were collected and assayed for IL-4 and IFN- $\gamma$  production by standard ELISA techniques using IL-4 or IFN- $\gamma$  monoclonal antibody pairs.

Briefly, wells of a 24-well microtitre plate were coated with a capture anti-IL-4 antibody. After incubation at room temperature for 1 hr, the wells were washed and biotinylated anti-IL-4 antibody was added to each well. Following incubation for a further 1 hr, the wells were washed and streptavidin-peroxidase conjugate was added to each well. After incubation for 30 mins, the wells were washed and then TMB substrate was added. The colour development was read at 450/620 nm in an ELISA plate reader. The level of IL-4 in unknown samples was quantitated by interpolation using a standard curve. A similar procedure was used for measurement of IFN- $\gamma$ .

The results shown in Fig. 1 A and B demonstrate that feeding *L. acidophilus* resulted in the suppression of IL-4 production in dose-dependent manner (Fig. 1A) whereas the production of IFN- $\gamma$  was enhanced (Fig 1B).

#### **Example 2: Effect of probiotic bacteria on nitric oxide (NO) secretion in saliva**

DBA/2 mice ( $n = 3$  to  $5$  per group) were fed *Lactobacillus acidophilus* and then challenged with *Candida albicans* as described in Example 3 below. At various times following oral infusion, saliva was collected after injection with pilocarpine to stimulate saliva flow. The concentration of NO in saliva was determined by the Griess reaction according to the method by Gree et al (1982) Anal. Biochem 126:131-138.

The data demonstrate that secretion of NO into saliva of animals fed *Lactobacillus acidophilus* (oral administration of  $5 \times 10^9$  CFU) was increased early

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after challenge with *Candida albicans* but was significantly reduced at a later stage (Figure 2).

**Example 3 Resistance to *C. albicans* following feeding with probiotic bacteria**

5 DBA/2 mice H2d male mice (n = 3-5 per group) were fed intragastrically every 2 days for 2 weeks with  $5 \times 10^9$  *L. acidophilus* in 0.2mL PBS. One day after the last feed mice were challenged with  $10^8$  *Candida albicans* blastospores by swabbing the oral cavity using a fine-tip sterile swab. At various days after infection the number of *C. albicans* in the oral mucosa was determined. The  
10 oral cavity (cheek, tongue and soft palate) was completely swabbed using a fine-tipped cotton swab. The cotton swab was placed in 1 mL PBS and the yeast cells were resuspended by mixing in a vortex mixer before culture of serial 10-fold dilutions on Sabourand dextrose agar supplemented with chloramphenicol at 37°C. The results were expressed as CFU/mL (Figure 5)

15 Mice fed *L. acidophilus* were more resistant to infection at all time points compared to control mice fed PBS. By day 6 the yeasts were almost completely cleared from mice fed *L. acidophilus*.

**Example 4: IL-4, IFN- $\gamma$  and IL-12 production**

A further study on responses to *Candida albicans* infection was  
20 conducted in the model described in Example 3.

*Candida* antigen was prepared from freshly cultured *Candida albicans* by sonication in a MSE Soniprep. The sonicate was centrifuged for 10 min at 2000g after which time the supernatant was collected and dialysed against PBS. After protein estimation, the solution was filtered sterilised and stored at -20°C  
25 until needed.

A single cell suspension of cervical lymph node (CLN) cells was prepared by teasing lymph node tissue through a sieve. The cells were collected in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS), washed twice by centrifugation and cultured at  $4 \times 10^6$  cells per well in the presence of 2.5 $\mu$ g/mL  
30 of *Candida* antigen in a 24-well plate for 3 days.

The culture supernatants were collected and assayed for IL-4, IL-12 and IFN- $\gamma$  by ELISA using matched antibody pairs and recombinant cytokines as

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standards (Pharmingen, San Diego, CA). The sensitivity of the cytokine ELISA was 31pg/mL and the results were expressed in net amounts from which the background was subtracted.

Samples of saliva were obtained according to the procedure described in  
5 example 2.

The results of IL-4 and IL-12 study are shown in Figure 3 (IL-12) and Figure 4 (IL-4).

The results of the IFN- $\gamma$  study are shown in Figures 6 (salivary IFN- $\gamma$ ) and 7 (cervical lymph node IFN- $\gamma$ ).

10 The control data shows a small poorly sustained response to oral infection with *Candida albicans*, with no detectable IFN- $\gamma$  until day 6 of the infection. The data in mice fed *L. acidophilus* indicates that there is no IFN- $\gamma$  response in uninfected mice but that there is an early response (day 2) when mice were challenged with *C. albicans*. Further, there was a more sustained  
15 IFN- $\gamma$  response after challenge with *C. albicans* than in the controls not fed *L. acidophilus*. This primary effect can still be seen in the nodes at day 15 and in saliva at day 8.

Not wishing to be bound by any particular mechanism of action, it seems that the mucosal surfaces are primed by the administration of *Lactobacillus* to  
20 secrete IFN- $\gamma$  following a challenge and in this model IFN- $\gamma$  operates to prevent conversion of the pathogen, *Candida albicans*, to the mycelial (invasive) form of the fungus and enhances cellular immunity and nitric oxide production.

#### **Example 5. Role of IFN- $\gamma$ and NO in resistance to infection**

To identify the immune parameters of protection, the levels of IFN- $\gamma$  were  
25 determined at various times following infection with *C. albicans*. As shown in Fig 8, there was a marked increase in the levels of IFN- $\gamma$  in saliva immediately and on day 1 after challenge with *C. albicans* compared with unimmunised mice. The levels were sustained over 15 days with significantly higher levels detected on day 10 and day 15 compared with those in unimmunised mice. Since nitric oxide  
30 production is associated with host defence in parasitic infection, quantitation of NO was performed following infection in two mouse strains sharing the same H2d MHC haplotype. In this experiment mice were infected with *C. albicans* and

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then followed by ip injection with an inhibitor of NO synthase, by injecting NG - monomethyl-L-arginine monoacetate (MNLA ) daily for 3 days after which time the clearance rate of yeasts was determined. As shown in Fig 9, mice treated with MNLA had delayed clearance of yeasts at various time points in the two  
 5 mouse strains compared with untreated mice, indicating that reduction in NO production is associated with resistance.

**Example 6: Enhanced clearance of non-typable *H influenzae* from the lungs of rats fed *L acidophilus***

DA rats ( 200- 250 gm, 8-10 weeks old, Animal Resource Centre, Perth,  
 10 WA) were fed intragastrically *L acidophilus* in 0.75 mL PBS (2.5 x 10<sup>10</sup> per rat) or PBS alone every 2 days for 7 days at which time the rats were immunised with formalin-killed *H influenzae* ( 5 x 10<sup>9</sup> per rat ) administered intralumenally (see groups below). Rats continued to be fed every 2 days for 2 weeks and then boosted with 50 µL of formalin killed *H influenzae* ( 5 x 10<sup>8</sup> per rat )  
 15 administered by the intratracheal route. After further feeding with *L acidophilus* for a further 7 days, the rats were challenged with 50 µL of live 5 x 10<sup>8</sup> *H influenzae* in the lung.

Immunisation groups (5 rats/group):

- 1 PBS ( intralumenal )
- 20 2 *H influenzae* ( intralumenal) + *H influenzae* ( intra-tracheal boost)
- 3 PBS (intralumenal) + *L. acidophilus* fed every 2 days
- 4 *H influenzae* ( intralumenal ) + *H influenzae* ( intratracheal boost) + *L. acidophilus* fed every 2 days

After 4 hrs post challenge, the rats were sacrificed and the levels of  
 25 colonisation in the lung was examined in bronchial lavage (BAL) and lung homogenate (LH). The level of clearance was determined by a plating serial 10-fold dilutions of the lavage fluid or lung homogenate onto chocolate agar plates. The results were expressed as number of colony forming units (CFU).

The results are shown in Table 1. Rats fed *L acidophilus* and  
 30 immunised with killed *H influenzae* were more resistance to infection *H influenzae* in the lungs. Furthermore, rats fed orally with repeated doses of *L acidophilus* were more resistant to infection than rats given a single bolus of *L*



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*acidophilus*, suggesting that enhanced clearance may be due to increased colonisation in the gut with *L. acidophilus* following repeated feeding.

Table 1 Increased clearance of *H. influenzae* in the lungs of rats fed *L. acidophilus*

Groups	BAL ( CFU/mL x 10 <sup>6</sup> )	LH (CFU/mL x 10 <sup>6</sup> )
PBS	26 ± 7.5	90.2 ± 29
<i>H. influenzae</i> + <i>H. influenzae</i> boost	4.6 ± 3.0	4.9 ± 1.2
PBS + <i>L. acidophilus</i>	0.7 ± 0.3	12.5 ± 5.7
<i>H. influenzae</i> + <i>H. influenzae</i> boost + <i>L. acidophilus</i>	0.19 ± 0.1	3.2 ± 2.1

5 **Example 7. Effect of *Lactobacillus acidophilus* ( VRI 011) on translocation of *Salmonella typhimurium* in mice following immunisation with heat killed *Salmonella* vaccine.**

Translocation of gram-negative bacteria across the gut epithelium can occur especially in subjects following post-operative surgery or gastrointestinal  
 10 infection. Left untreated it can lead to endotoxiemia. In this example, the effect of feeding *L. acidophilus* on the translocation of gut pathogen *Salmonella typhimurium* is examined.

Female BALB/c mice 6-8 weeks old were obtained from the Animal Resource Centre, Perth, WA. *Lactobacillus acidophilus* strain (VRI 011 ) and the  
 15 pathogen *Salmonella typhimurium* were obtained from the School of Microbiology and Immunology Type Culture Collection, University of New South Wales.

Mice were given 5 doses of *L. acidophilus* (VRI 011) (1 x 10<sup>8</sup> cfu per dose) or PBS over a 2 week period after which time mice were immunised  
 20 intragastrically with a killed oral *Salmonella* vaccine. Two weeks after immunisation, the mice were sacrificed and the spleens collected for enumeration of *L. acidophilus* and *S. typhimurium*.

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Mice fed *L acidophilus* (VRI 011) before or simultaneously with a killed oral *S typhimurium* vaccine have better protection against translocation of *S typhimurium* following live challenge via the oral route compared with mice fed PBS (Table 1). Immunisation with a killed oral vaccine led to decreased

5 translocation of *S typhimurium* to the spleen but it was further enhanced by feeding *L acidophilus*. Thus translocation of pathogenic bacteria through the gut epithelium can be prevented by oral administration of probiotic.

**Example 8: Effect of *Lactobacillus fermentum* on translocation of pathogenic *S typhimurium* from the gut of mice vaccinated with killed**

10 **oral Salmonella vaccine**

BALB/c female mice ( 6-8 weeks old ) were fed  $10^8$  *L fermentum* VRI012 (obtained from University of New South Wales, School of Microbiology and Immunology Culture Collection, Sydney, Australia) before or co-administered with  $10^8$  killed *S typhimurium* vaccine four times over a 2 week

15 period. Two weeks after oral administration, the mice were challenged with live *S typhimurium* administered by the oral route. After sacrifice, the level of colonisation with *S typhimurium* in the spleen was determined. Table 2 showed that low levels of translocation occurred in mice fed *L fermentum* prior to immunisation or co-administered with *S typhimurium* compared with mice

20 fed PBS.

Table 2 *L fermentum* VRI012 enhanced the inhibition of translocation of *S typhimurium* in mice immunised with killed *S typhimurium* vaccine.

Group treatment	Log CFU/g wet wt of spleen ( $\pm$ SEM)
PBS ( Control)	$10.12 \pm 0.54$
Killed <i>S typhimurium</i>	$5.5 \pm 0.27$ $p < 0.05$ vs Control
<i>L fermentum</i> KLD ( before )	$3.8 \pm 0.12$ $p < 0.05$ vs Control
<i>L fermentum</i> KLD (co-administered)	$4.5 \pm 0.22$ $p < 0.05$ vs Control

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**Example 9. Effect of *L. fermentum* on clearance of NTHi bacteria**

Groups of 6 male, specific pathogen free, dark agouti (DA) rats were given a single dose of PBS or live *L. fermentum* ( $2.5 \times 10^{10}$ ) directly into the duodenal lumen after exposure of the intestine by laparotomy. Twenty-one days later the rats were infected intratracheally with  $5 \times 10^8$  live Non-typeable *Haemophilus influenzae* (NTHi). Four hours later the rats were killed by pentobarbitone overdose. The lungs were lavaged with 10 mL PBS to obtain broncho-alveolar lavage fluid (BAL). The lavaged lungs were then homogenised in 10mL of PBS. Twenty microlitres of ten-fold serial dilutions of BAL and lung homogenate (LH) were plated on chocolate agar plates and incubated in a 10% CO<sub>2</sub> incubator at 37°C overnight. Colonies were counted and the number of live bacteria in the BAL and LH preparations determined. The recovery of live NTHi bacteria, presented as colony forming units (CFU), was as follows:

Table 3.

Rats dosed with:	BAL CFU ( $10^6$ )	LH ( $10^6$ )
PBS	$3.67 \pm 1.30$	$103.5 \pm 34.2$
<i>L. fermentum</i>	$0.49 \pm 0.19$	$0.82 \pm 0.37$ p = 0.017*

\* When compared to PBS group

These data show that a single dose of *L. fermentum* delivered to the gut causes enhanced clearance of a subsequent acute NTHi respiratory infection.

**Example 10. Effect of *L. acidophilus* on clearance of *H. pylori***

Two groups of 5 female C57BL/6 mice were infected with *H. pylori* (Sydney strain 1) by administration of approx.  $10^9$  live *H. pylori* on three consecutive days (days 1,2 and 3) by gavage. On days 28, 30, 32, 35, 37 and 39 mice were dosed by gavage with live *L. acidophilus* ( $5 \times 10^9$  in 0.2 mL water) or water (control group).

Twenty-one days later (day 50) mice were killed by pentobarbitone overdose (administered intra-peritoneally) and their stomachs removed. The stomach was dissected into two equal halves and one half was placed in 1mL of deionised water and homogenised. Serial 10-fold dilutions of stomach homogenate were plated out on chocolate agar plates containing fungizone, vancomycin, bacitracin and nalidixic acid. After incubation for 3 days at 37°C under microaerophilic conditions, the colonies were counted and the total

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number of live *H. pylori* in the original stomach homogenate determined. The results for recovery of live *H. pylori* in the stomach, expressed as colony-forming units (CFU), are shown in the following table.

Table 4:

5

Mice dosed with:	Number of <i>H. pylori</i> in stomach homogenate (10 <sup>4</sup> CFU)
Deionised water	252 ± 44
<i>L. acidophilus</i> in water	105 ± 14

The two groups are significantly different (P = 0.013)

These results show that mice dosed with *L. acidophilus* have a lower level of *H. pylori* infection of the stomach than do control mice. This is an example of a therapeutic effect of *L. acidophilus* on a pre-established *H. pylori* infection.

#### 10 Example 11: Anti-bacterial therapy (Metronidazole) of *Helicobacter pylori* infection in mice pre-treated with probiotic

*H. pylori* infected mice (5 per group) were treated with *Lactobacillus acidophilus* (1x10<sup>9</sup> per animal) by oral feeding 3 times a week for 2 weeks prior to treatment with Metronidazole (0.08mg/animal) for 1 week.

15 The following results demonstrate that highly significant eradication of bacteria from the stomach was noted in mice pre-treated with probiotic and followed by antibiotic therapy (70%, p=0.009), those treated with probiotic alone (58%, p=0.013), and those treatment with metronidazole alone (55%, p,0.025), when compared with the saline-treated control group.

20 Table 6:

Treatment Group	<i>H. pylori</i> colonisation (x 10 <sup>5</sup> CFU/animal)	% eradication
Saline	25 ± 4.3	0
<i>L. acidophilus</i>	10.5 ± 1.4	58 <sup>a</sup>
Metronidazole	11.4 ± 2.5	55 <sup>b</sup>
<i>L. acidophilus</i> + Metronidazole	7.5 ± 2.6	70 <sup>c</sup>

a, p<0.01; b, p<0.02; c, p<0.005 compared with control values

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The above examples demonstrate that probiotic bacteria, by driving a particular cytokine balance (from gut derived but mucosally re-located T cells), create an environment less favourable for particular microbial growth.

Although the present invention has been described with reference to  
5 preferred embodiments it will be understood that variations in keeping with the inventive concept described herein are also contemplated.

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**CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:-**

- 1     A method of prophylactic or therapeutic treatment of chronic or acute  
infection, or of undesirable microbial colonisation, of a mucosal surface, other  
than intestinal mucosal surface, comprising the administration of an effective  
5   amount of a probiotic as herein defined, or of a probiotic-containing composition,  
to a subject in need thereof.
- 2     A method according to claim 1 wherein the mucosal surface is selected  
from the group consisting of oral, nasopharyngeal, respiratory, gastric,  
reproductive and glandular.
- 10    3     A method of prophylactic or therapeutic treatment of a chronic or acute  
disorder of mucosal surface of the respiratory tract, comprising the  
administration of an effective amount of a probiotic as herein defined, or of a  
probiotic-containing composition, to a subject in need thereof.
- 4     A method according to claim 3, wherein the mucosal surface is oral  
15    mucosa.
- 5     A method according to claim 3, wherein the mucosal surface is lung  
mucosa.
- 6     A method according to any one of the preceding claims, wherein the  
probiotic as herein defined, or the probiotic-containing composition, is  
20    administered to the gastric or to the intestinal mucosal surface.
- 7     A method of prophylactic or therapeutic treatment of a chronic or acute  
disorder of a mucosal surface caused by disturbance in cytokine balance or lack  
of an appropriate T cell immune response, comprising the administration of an  
effective amount of probiotic bacteria, or a probiotic bacteria-containing  
25    composition, to a subject in need thereof.
- 8     A method according to claim 6 or claim 7 wherein the mucosal surface is  
selected from the group consisting of oral, nasopharyngeal, respiratory, gastric,  
intestinal, reproductive and glandular.
- 9     A method according to any one of claims 3 to 8, wherein the disorder is  
30    an infection or inappropriate microbial colonisation.
- 10    A method according to claim 9 wherein the infection is a bacterial  
infection caused by one or more bacteria selected from the group consisting of

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*Pseudomonas* species, *Streptococcus* species, *Staphylococcus* species, *Candida* species, *Helicobacter* species and *Haemophilus* species.

11 A method according to claim 10 wherein the one or more bacteria are selected from the group consisting of non-typable *Haemophilus influenzae*,  
5 *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Staphylococcus albus*, *Staphylococcus aureus*, *Candida albicans* and *Helicobacter pylori*.

12. A method according to claim 9, wherein the disorder is an infection or inappropriate colonisation by a virus.

13. A method according to claim 12, wherein the virus is selected from the  
10 group consisting of Epstein-Barr virus (EBV), cytomegalovirus (CMV), Ross River virus (RRV), herpes-type virus and the like.

14. A method according to claim 9 or claim 12, wherein the disorder is chronic fatigue syndrome (CFS).

15 A method according to any one of claims 7 to 14 wherein the cytokine is  
15 interferon- $\gamma$ , interleukin-4 and/or interleukin-12.

16 A method according to any one of claims 9 to 15, wherein the appropriate T cell immune response is Th1 response.

17 A method according to any one of claims 1 to 16, wherein the probiotic as herein defined is, or probiotic-containing composition contains, a *Lactobacillus*  
20 or a *Mycobacterium* species.

18 A method according to claim 17, wherein the probiotic as herein defined is, or probiotic-containing composition contains, *Lactobacillus acidophilus*, *Lactobacillus fermentum*, *Lactobacillus casei* and/or *Mycobacterium vaccae*.

19 A method according to claim 17 or claim 18 wherein the probiotic as herein  
25 defined, or probiotic-containing composition, includes viable organisms.

20 A method according to any one of claims 1 to 19, wherein the effective amount is in the range of about  $10^8$  to  $10^{12}$  CFU

21 A method according to claim 20 wherein the effective amount is about  $10^{11}$  CFU.

30 22 A method according to any one of claims 1 to 21, wherein the probiotic as herein defined or probiotic-containing composition is in a solid dosage form.

23 A method according to claim 22, wherein the dosage form is a tablet or a capsule.

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24 A method according to any one of claims 1 to 23, further comprising the administration of one or more pharmaceutically active agents.

25 A method according to claim 24, where the one or more pharmaceutically active agents is an antibiotic.

5 26 A method according to claim 24 or claim 25, wherein the probiotic as herein defined, or the probiotic-containing composition, is administered prior to, simultaneously with or subsequent to one or more pharmaceutically active agents.

27 A method according to any one of claims 1 to 26, wherein the site of  
10 administration of the probiotic as herein defined, or of the probiotic-containing composition, is distal to the mucosal surface having the disorder.

28 A method according to claim 27, wherein the probiotic as herein defined, or the probiotic-containing composition, is administered to the intestinal mucosal surface in the treatment of oral, nasopharyngeal, gastric, respiratory tract,  
15 reproductive tract or glandular mucosal infection.

29 A method of altering cytokine balance at a mucosal surface in a subject comprising the administration of an effective amount of a probiotic as herein defined, or of a probiotic-containing composition, to a subject in need thereof.

30 A method of inducing a Th1 cellular immune response at a mucosal  
20 surface in a subject comprising the administration of an effective amount of a probiotic as herein defined, or of a probiotic-containing composition, to a subject in need thereof.

31 A method of enhancing the secretion of interferon- $\gamma$  at a mucosal surface in a subject comprising the administration of an effective amount of a probiotic  
25 as herein defined, or of a probiotic-containing composition, to a subject in need thereof.

32 A method of reducing the secretion of interleukin-4 at a mucosal surface in a subject comprising the administration of an effective amount of a probiotic as herein defined, or of a probiotic-containing composition, to a subject in need  
30 thereof.

33 A method of restoring normal cytokine balance at a mucosal surface in a subject comprising the administration of an effective amount of a probiotic as



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herein defined, or of a probiotic-containing composition, to a subject in need thereof.

34 A method of priming mucosal surface for immunotherapy comprising the administration of an effective amount of a probiotic as herein defined, or of a  
5 probiotic-containing composition, to a subject in need thereof.

35 A method according to claim 34, wherein immunotherapy consists in administration of a therapeutic or prophylactic vaccine.

36 A method according to claim 34 or claim 35, wherein the probiotic as herein defined, or the probiotic-containing composition, is administered before  
10 or simultaneously with the vaccine.

37 A method according to claim 35 or claim 36, wherein the probiotic as herein defined, or the probiotic-containing composition, is additionally administered after the vaccine.

38 A pharmaceutical composition suitable for prophylactic or therapeutic  
15 treatment of chronic or acute disorder of a mucosal surface comprising an effective amount of a probiotic as herein defined, or of a probiotic-containing composition.

39 A pharmaceutical composition according to claim 38, wherein the probiotic as herein defined is, or probiotic-containing composition contains, a  
20 *Lactobacillus* or a *Mycobacterium* species.

40 A pharmaceutical composition according to claim 39, wherein the probiotic as herein defined is, or probiotic-containing composition contains, *Lactobacillus acidophilus*, *Lactobacillus fermentum*, *Lactobacillus casei* and/or *Mycobacterium vaccae*.

25 41 A pharmaceutical composition according to any one of claims 38 to 40, wherein the probiotic as herein defined, or probiotic-containing composition includes, viable organisms.

42 A pharmaceutical composition according to any one of claims 38 to 41, wherein the composition contains about  $10^8$  to about  $10^{12}$  CFU

30 43 A pharmaceutical composition according to claim 42 wherein the composition contains about  $10^{11}$  CFU.

44 A pharmaceutical composition according to any one of claims 38 to 43, wherein the pharmaceutical composition is in a solid dosage form.

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- 45     A pharmaceutical composition according to claim 44, wherein the dosage form is a tablet or a capsule.
- 46     Method of prophylactic or therapeutic treatment of a symptom and/or syndrome associated with chronic or acute infection, or with undesirable
- 5     microbial colonisation or reactivation, of a mucosal surface, comprising the administration of an effective amount of an agent capable of non-specific activation of the common mucosal system to a subject in need thereof.
47.     A method according to claim 46, wherein the symptom and/or syndrome is chronic fatigue syndrome.
- 10     48.     A method according to claim 46 or claim 47, wherein the agent is a probiotic as herein defined, or a probiotic-containing composition.
49.     A method according to claim 48, wherein the probiotic as herein defined is, or the probiotic-containing composition comprises, viable intact organisms.
50.     A method according to any one of claims 46 to 49, wherein the subject is
- 15     selected from the group consisting of an EBV positive athlete, a subject with sudden onset CFS, a subject with protracted fatigue following exercise, a subject with low level of salivary IgA or IgA1 and a subject with documented infectious mononucleosis.

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## A. IL-4

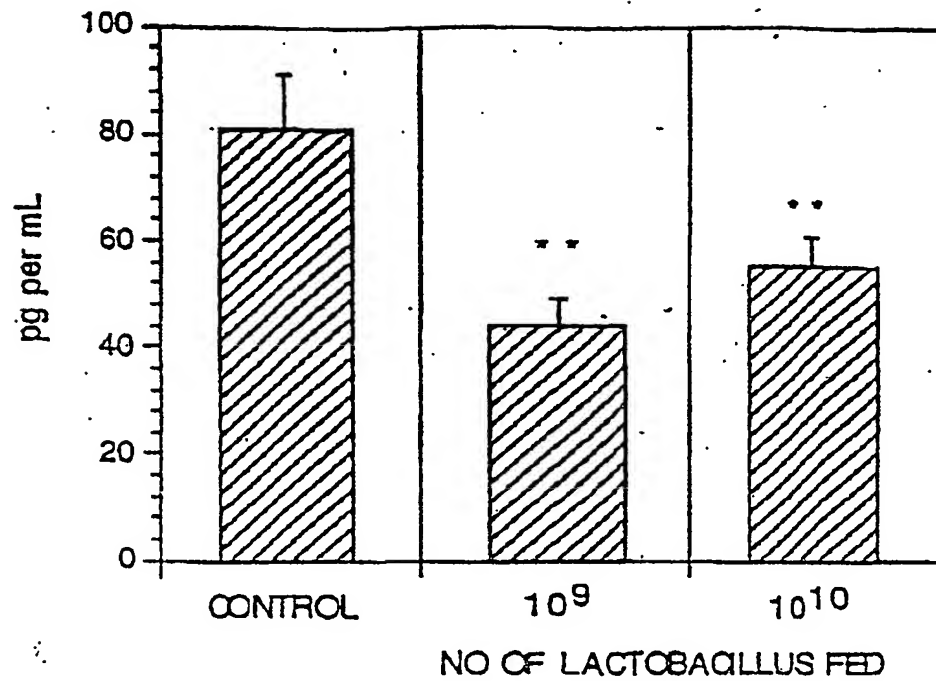
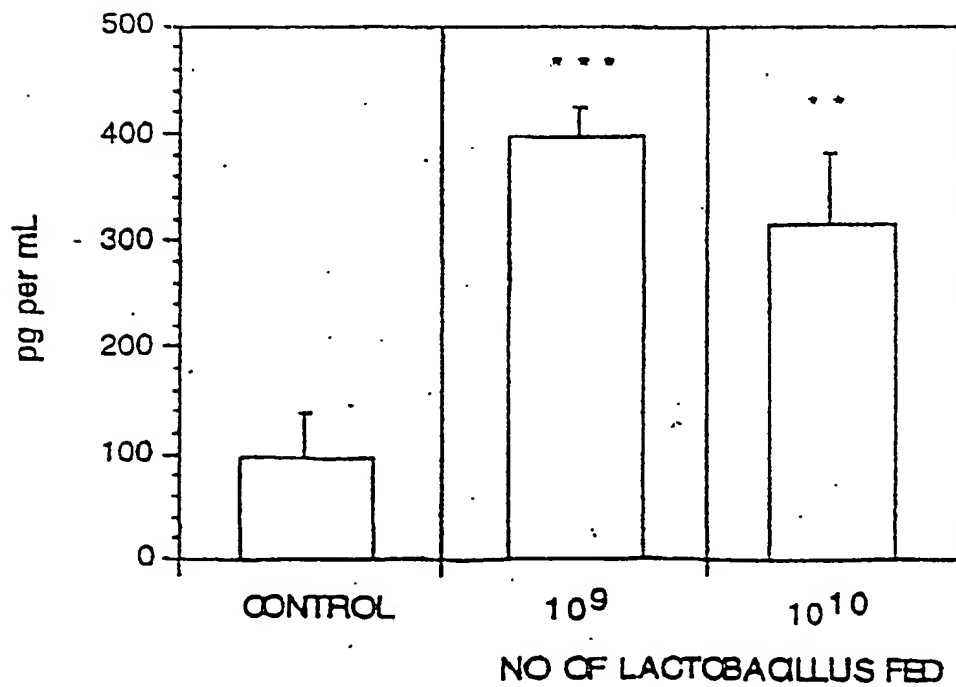
B. IFN- $\gamma$ \*\*,  $p < 0.01$ \*\*\*,  $p < 0.001$ 

Figure 1

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NO in saliva

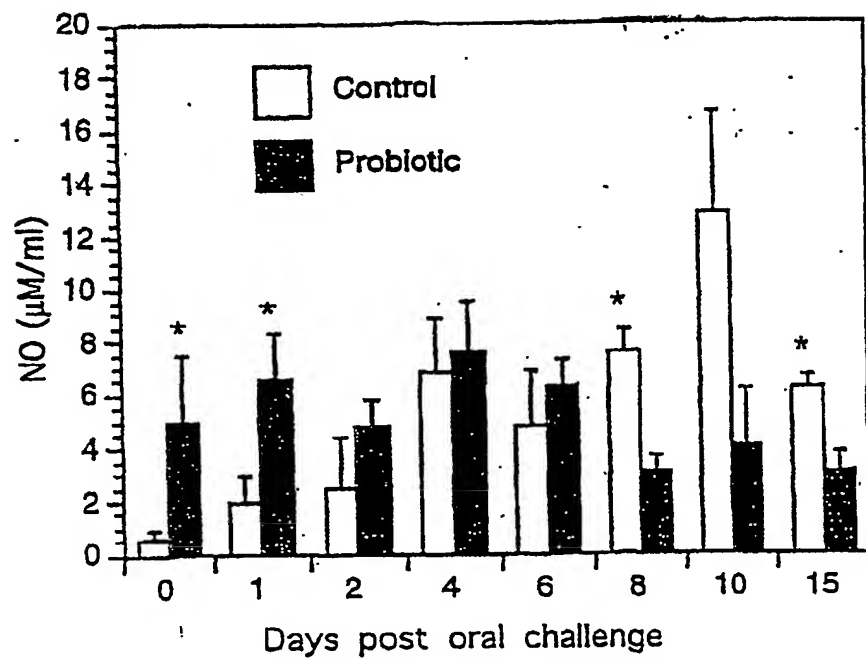


Figure 2

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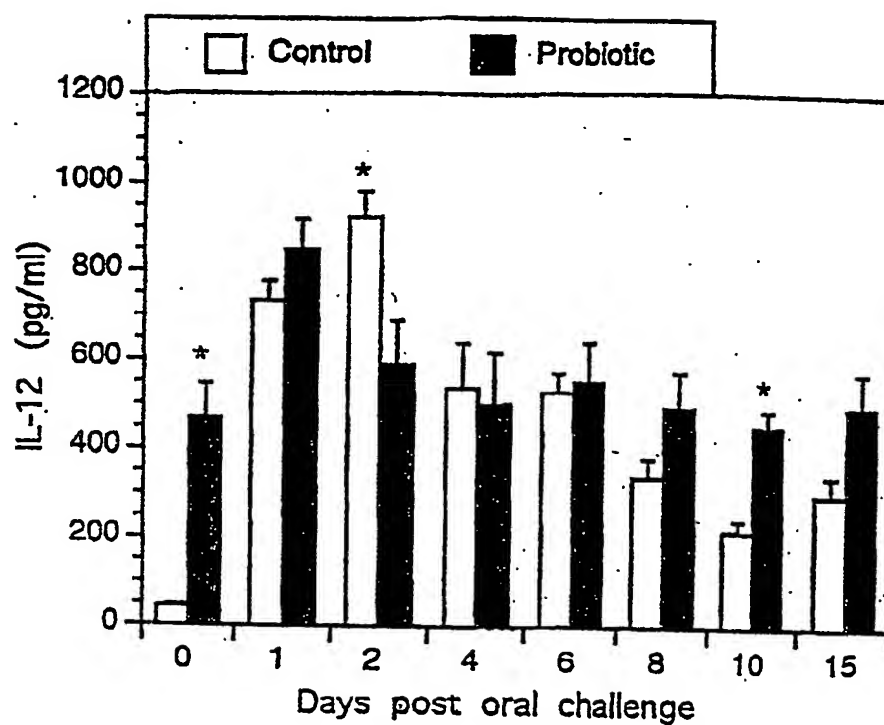


Figure 3

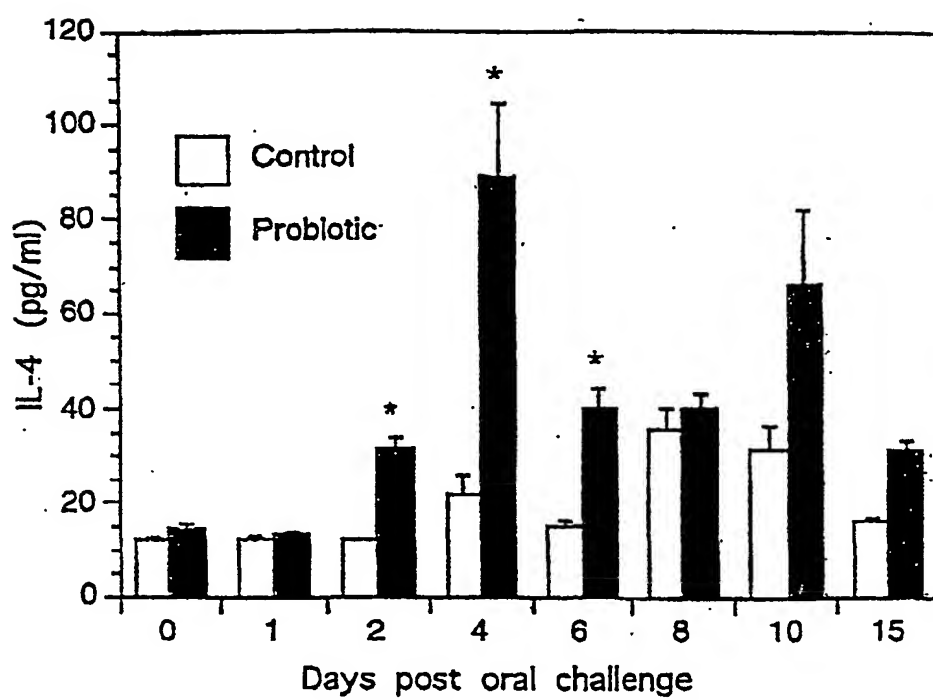
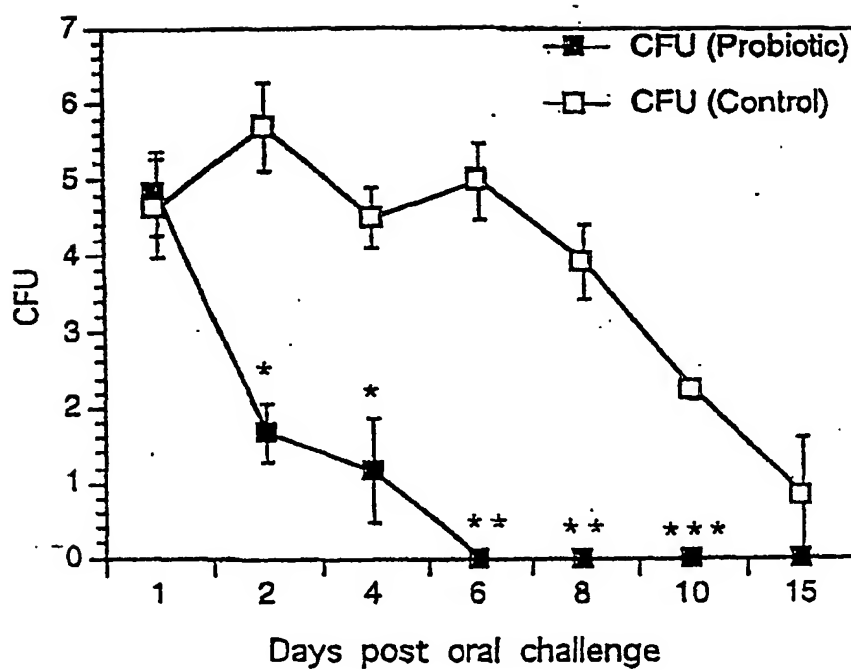


Figure.4

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\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$

Figure 5

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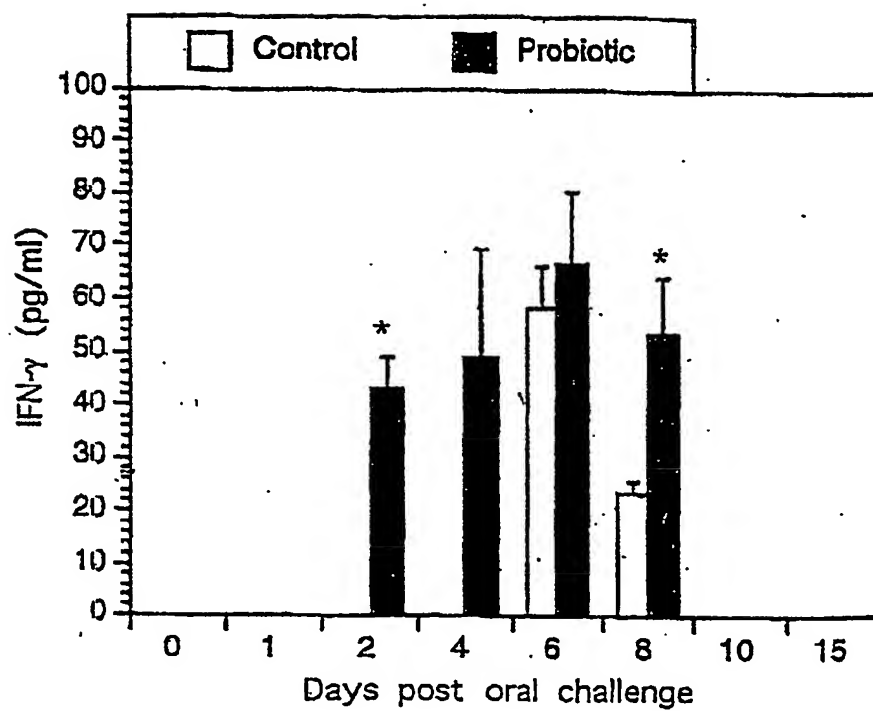
Saliva IFN- $\gamma$ Saliva IFN- $\gamma$ 

Figure 6



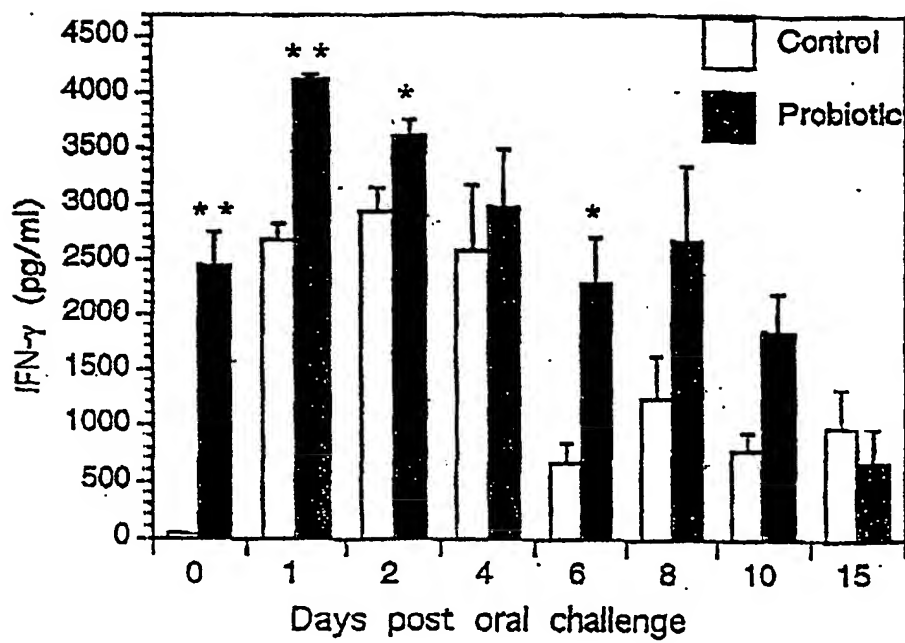
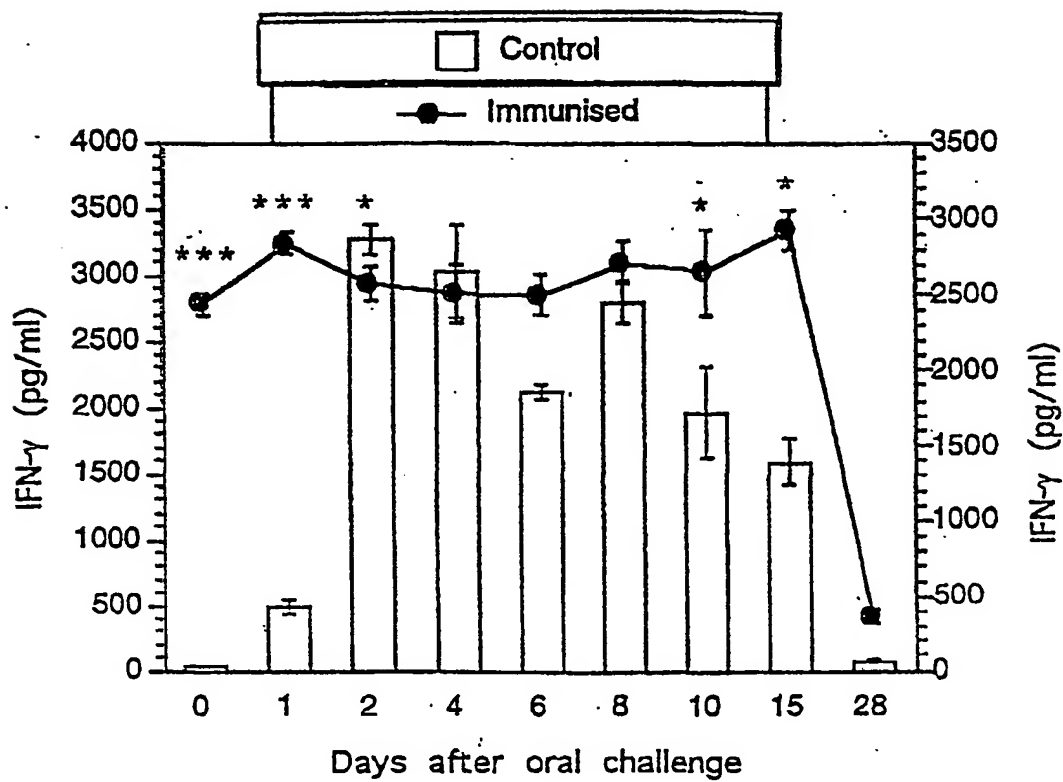


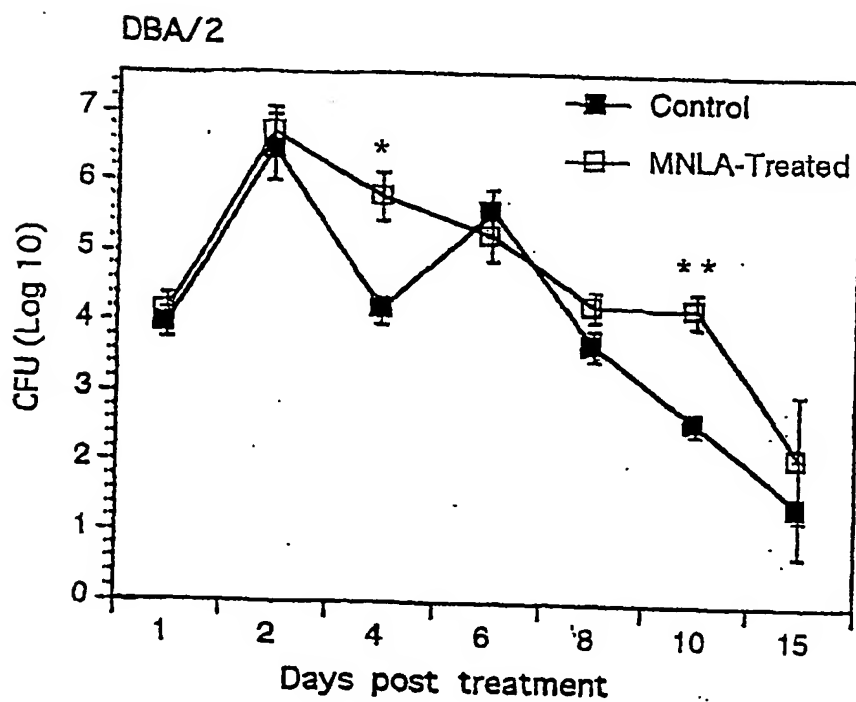
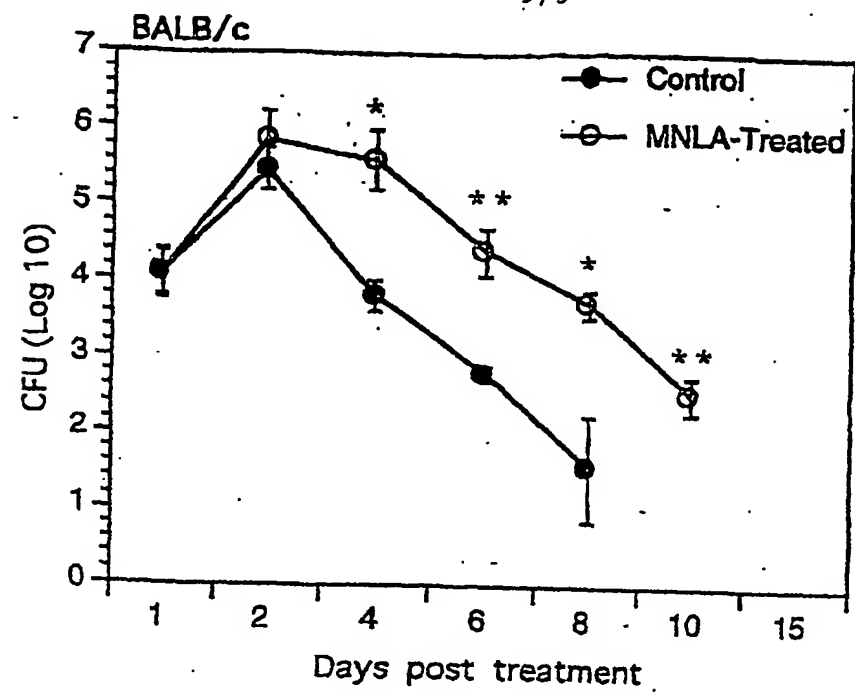
Figure 7



\* P < 0.05, \*\*\* P < 0.0001

Figure 8

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\*  $P < 0.05$ , \*\*  $P < 0.01$

Figure 9

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU01/00726

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>		
Int. Cl. <sup>7</sup> : A61K 35/74, 39/02, 39/04, 39/07, A61P 31/04, 1/00, 1/04, 1/14, 11/00, 15/02		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b>		
Minimum documentation searched (classification system followed by classification symbols) A61K with keywords as below		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched AU: IPC as above		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) WPAT: Probiotic, lactobacillus(w)acidophilus, casei, mycobacterium vaccae and A61K		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99/17788 A (Abbott Laboratories) 15 April 1999 Whole document, especially pages 1-12	1-50
X	WO 99/07393 A (Teodorescu) 18 February 1999 pages 1-5 and 22	38-41, 44,45
X	WO 98/55131 A (Essum AB) 10 December 1998 Whole document	6-11, 17-23, 29-33, 38-45,
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C <input checked="" type="checkbox"/> See patent family annex		
<p>* Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier application or patent but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p>		
Date of the actual completion of the international search 23 July 2001		Date of mailing of the international search report 26 July 2001
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustalia.gov.au Facsimile No. (02) 6285 3929		Authorized officer G.J. McNEICE Telephone No : (02) 6283 2055

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU01/00726

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98/26790 A (Stanford Rook Ltd.) 25 June 1998 Entire document	7-9, 14, 17-23, 29-50
X	WO 97/36603 A ( Commonwealth Scientific And Industrial Research Organisation ) 9 October 1997 Entire document	1-23, 27-33, 38-50
X	WO 98/23727 A ( Bio K + International Inc. ) 4 June 1998 Entire document, especially pages 1-12, 18-20	1-23, 27-33, 38-50
X	US 4591499 A (Linn, L.L. et al) 27 May 1986 Columns 1-8	1-3, 7-11, 15-23, 27-33, 38-46
X	EP 353581 A (Dr. A. Tosi Farmaceutici S.r.l. ) 7 February 1990 Pages 1-6	7-11, 17-23, 27-33, 38-46,
X	EP 199535 A (The New England Medical Centre Hospitals Inc.) 29 October 1986 Pages 1-20	6-11, 17-23, 27, 29-33, 38-49

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

International application No.  
**PCT/AU01/00726**

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report			Patent Family Member			
WO	9917788	NONE				
WO	9907393	AU	87540/98			
WO	9855131	AU	80460/98	EP	1005353	NO 995794
		SE	9702083			
WO	9826790	AU	77355/98	BR	9713959	CN 1246056
		EP	951289	FI	991384	NO 992957
		HU	200001602			
WO	9736603	AU	21451/97			
WO	9823727	AU	51137/98	EP	951531	
US	4591499	NONE				
EP	353581	IT	1227154	JP	2174675	US 5176911
		ZA	8905936			
EP	199535	AU	56112/86	DK	1701/86	FI 860194
		FI	923829	JP	61280433	NO 861483
		US	4839281	US	5032399	
						END OF ANNEX